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# Cooperative Binding Interactions Required for Function of the Ty1 Sterile Responsive Element

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**The Ste12p transcription factor controls the expression of Ty1 transposable element insertion mutations and genes whose products are required for mating in *Saccharomyces cerevisiae*. The binding site for Ste12p is a consensus DNA sequence known as a pheromone response element (PRE). Upstream activating sequences (UASs) derived from known Ste12p-dependent genes have previously been characterized to require either multiple PREs or a single PRE coupled to a binding site for a second protein. The Ste12p-dependent UAS from Ty1, called a sterile response element (SRE), is of the second type and is comprised of a PRE and an adjacent TEA (TEF-1, Tec1, and AbaA motif) DNA consensus sequence (TCS). In this report, we show by UV cross-linking analysis that two proteins, Ste12p and a protein with an apparent size of 72 kDa, directly contact the Ty1 SRE. Other experiments show that Tec1p is required for formation of the Ty1 SRE protein-DNA complex and is physically present in the complex. These results establish a direct role for Tec1p in the Ty1 SRE and yet another set of combinatorial interactions that achieve a qualitatively distinct mode of transcriptional regulation with Ste12p.**

The Ste12p transcription factor is important for two distinct life cycle transitions of *Saccharomyces cerevisiae*. These transitions are from a vegetative form to either a mating-competent or pseudohyphal differentiated state. The **a** and α haploid cell types differentiate into mating-competent forms upon exposure to the mating pheromone from the opposite cell type (4). One aspect of this differentiation is the transcriptional induction of genes whose products execute functions associated with multiple events during the mating process. These include genes encoding pheromone, receptor, and other components of the pheromone-induced signal pathway and those that mediate growth arrest, nuclear migration, cell fusion, and adaptation (reference 37 and references therein). The common feature of these genes is the presence of pheromone response elements (PREs) within their promoter regions (19, 35). The consensus sequence (TGAAACA) is the binding site for Ste12p (7). The **a**/α diploid cell type differentiates into a pseudohyphal form under conditions of nitrogen deprivation (12). Ste12p is required for this transition, but the target genes for this response have yet to be identified (23).

Evidence to date shows that Ste12p binds poorly to a single PRE but will bind cooperatively to multiple PREs or to a single PRE when it couples to binding sites for other proteins (7, 9, 20, 28). These different associations allow flexibility in DNA recognition and underlie the qualitative and quantitative differences in the expression of various Ste12p-dependent genes. Three examples of Ste12p-responsive genes illustrate these differences. *FUS1* has multiple upstream PREs and is completely dependent on Ste12p for basal and pheromone-induced expression (13). The *STE2* promoter has three PREs, one adjacent to a P box, which is the binding site for Mcm1p (17–19, 26, 35). While pheromone-induced activity of the *STE2* promoter is completely dependent on Ste12p, there is residual basal activity in the absence of Ste12p that is attributable to Mcm1p (15). The *KAR3* promoter has two near consensus PREs, one of which is adjacent to a putative Kar4p binding site. Vegetative expression of *KAR3* is independent of either Ste12p or Kar4p, but pheromone-induced expression is dependent on both transcriptional regulators (20).

Ste12p also regulates the expression of Ty1 insertion mutations such as *CYC7-H2* (10). An element within the Ty1 regulatory region was identified as sufficient to support Ste12p-dependent activation of adjacent gene expression (3). Various analyses of this Ste12p-responsive element (SRE) implicated two regions as important for its upstream activation sequence (UAS) function and for its complex formation with Ste12p (3, 9, 22, 24). One of these regions is the PRE; the other encompasses a sequence (CATTCT) that matches a TEA (TEF-1, Tec1, and AbaA motif) DNA consensus sequence (TCS) (9, 22, 24).

Function of the Ty1 SRE as a UAS was found also to depend on the expression of Tec1p (22). The predicted amino acid sequence of Tec1p has a TEA domain that defines a DNA binding motif common to Tec1p, the simian virus 40 enhancer factor, TEF-1, and aspergillus AbaA (1, 21). Together these observations lead to the prediction that, in addition to Ste12p, Tec1p could be a component of the Ty1 SRE protein-DNA complex. In this report, we show that a protein in addition to Ste12p directly contacts the Ty1 SRE DNA. We also provide evidence that Tec1p is required for formation of, and is physically present in, this multiprotein-DNA complex. Ty1 SRE function has recently been found to be induced in diploid cells by the same conditions that induce pseudohyphal differentiation (25). Therefore, we expect that the combinatorial interactions of Tec1p with Ste12p at the Ty1 SRE are similar to those that occur in the promoters of genes whose products mediate the transition from vegetative to pseudohyphal form.

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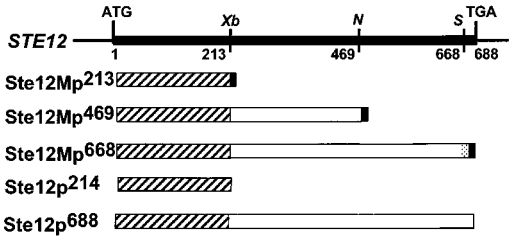


FIG. 1. Schematic representation of the *STE12* DNA locus and of Ste12p and Ste12p derivatives. The line diagram at the top represents the *STE12* coding region (thick line) and flanking sequences (thin lines). Restriction sites marking the junction points for recombinant DNAs encoding different epitope-tagged and/or truncation derivatives of Ste12p are shown above the line (*Xb*, *Xba*I; *Nc*, *Nco*I; *S*, *Sac*I). Codon positions for these junctions are numbered below the line. The boxes represent different protein forms of Ste12p. The hatched regions represent the N-terminal DNA binding domain of Ste12p. The solid regions represent the 11-amino-acid Myc epitope tag. The stippled region in Ste12Mp$^{668}$ represents 10 amino acids derived from Ste11p (9).

## MATERIALS AND METHODS

**Strains and yeast genetic procedures.** Strain E929-6C (*MAT***a** *cyc1 CYC7-H2 can1 leu2-3*, *112 trp1-*Δ*1 ura3-52*) and its isogenic derivatives E929-6C-6 (*ste12-*Δ*2::LEU2*), E929-6C-7 (*ste12-*Δ*1::URA3*), and E929-6C-22 (*bar1*Δ*::LEU2*) have been described previously (3, 39). Strain E929-6C-65 was constructed by gene replacement using the *tec1*Δ*::URA3* allele isolated as an *Xma*I-*Xma*I fragment of pCI-13 (21, 32). Procedures for growth and genetic manipulation of yeast strains were as described by Sherman et al. (34). The LiCl procedure was used for all yeast transformations (16).

**Plasmids, oligonucleotides, and recombinant DNA procedures.** Plasmids pNC247, pNC248, pGA1635, and pGA1841 were used to express Ste12p or different truncation derivatives in vivo. Plasmid pNC247 is a pNC160 (*CEN3 TRP1*)-based plasmid with the *STE12M-668* allele that encodes Ste12p amino acids 1 to 668 fused to 10 amino acids from Ste11 followed by an 11-amino-acid Myc epitope tag (Fig. 1, Ste12Mp$^{668}$) (9, 30). pNC247 is identical to the previously described plasmid pNC228 except for a *Sac*I restriction site in the *STE12* 5′ flanking region (9). Plasmid pNC248 is a YEp24 (2μm *URA3*)-based plasmid that carries the same *STE12M-668* allele as described for pNC247. Plasmid pGA1635 (2μm, *URA3*), with the *STE12M-469* allele that encodes Ste12p amino acids 1 to 469 fused to an 11-amino-acid epitope tag (Fig. 2, Ste12Mp$^{469}$), has been described elsewhere (9). Plasmid pGA1841 is a pNC161 (*CEN3 TRP1*)-based plasmid with the *TPI1-STE12M-213* allele that overexpresses Ste12p amino acids 1 to 213 linked to an 11-amino-acid Myc epitope tag (Fig. 1, Ste12Mp$^{213}$) (provided by G. Ammerer, University of Vienna) (30).

Plasmids pMP101 and pNC292 were used as templates for coupled in vitro transcription-translation synthesis of the full-length Ste12p or the N-terminal 214 amino acids of Ste12p (Fig. 1, Ste12p$^{688}$ and Ste12p$^{214}$, respectively). Plasmid pMP101 contains a T7 promoter and an encephalomyocarditis virus 3C leader fused to the *STE12* coding region (27, 28). pNC292 was constructed from pMP101 by digestion with *Xba*I, filling in the sticky ends, and religation. These manipulations create a +1 frameshift which adds an Asp codon followed by a TAG stop codon immediately after the *STE12* Ser 214 codon.

Plasmid pMOH4 for expression of the Tec1-Gal4p$^{AD}$ fusion used in these studies was kindly provided by K. Doi and K. Matsumoto (Nagoya University, Nagoya, Japan). pMOH4 was recovered from a pACT-based yeast genomic library during the course of a one-hybrid screen for genes encoding proteins that bind to the *MSG5* promoter (5, 6). The hybrid is a Tec1p polypeptide that lacks 56 amino acids from its N terminus fused in frame with the Gal4p activation domain (6).

UAS elements used for DNA probes and reporter gene constructs were derived from synthetic DNAs. Complementary oligonucleotides corresponding to the two strands of the wild-type Ty1 SRE (SRE-Wt) were synthesized and annealed to obtain a Ty1 SRE that encompasses the DNase I-protected region of the UAS as reported by Company et al. (3). The SRE-TCS* and SRE-PRE* mutant oligonucleotides have the same sequence as the wild-type SRE except for four mutations in the footprint region that encompasses the TCS or two mutations within the PRE, respectively (3) (Fig. 2). The evidence that delimits the sequences encompassing the *STE2* and *FUS1* UAS elements has been described elsewhere (9, 29) (Fig. 2). All oligonucleotides were made by automated synthesis at the oligonucleotide facility in the Department of Pathology at the University of North Carolina.

Plasmids pGA1696, pGA1702, pGA1706, pNC344, and pNC345 are *lacZ* reporter plasmids without or with different UASs. Plasmid pGA1696 carries the promoter-disabled *CYC1::lacZ* allele with *Bgl*II and *Xho*I cloning sites upstream of the *CYC1* TATA. Plasmids pGA1702 and pGA1706 contain the *STE2* or *FUS1* UAS, respectively, inserted into the *Xho*I site of pGA1696 (Fig. 2 and 3) (9, 29). Plasmids pNC343A and pNC343B were constructed by inserting the synthetic SRE-Wt at the *Xho*I site of pGA1696 and represent the two orientations of the inserted element (Fig. 2 and 3). Plasmids pNC344 and pNC345 have the synthetic SRE-TCS* and SRE-PRE*, respectively, inserted at the *Xho*I site of pGA1696 (Fig. 2 and 3).

Either pNC188 or pNC311 was used for the preparation of SRE probe DNAs. pNC188 has been described by Company et al. (3). The *Pst*I-*Sal*I end-repaired fragment from pNC188 was subcloned into the *Pst*I-*Hin*cII sites of pUC118 to generate pNC311 (36). The *Hin*dIII-*Xba*I fragment of pNC311 was used as probe DNA for the DNase I protection assay.

All recombinant DNA manipulations were carried out according to standard procedures (33). Enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or U.S. Biochemicals.

**Reporter gene assays.** The expression of different *lacZ* reporter genes was assessed by measuring the amount of β-galactosidase activity in yeast whole-cell extracts as described previously (29).

**Preparation of yeast whole-cell extracts.** Strains E929-6C-7, E929-6C-6/pNC247, E929-6C-6/pNC248, E929-6C-6/pGA1635, and E929-6C-6/pGA1841 were grown in liquid culture, using media appropriate for the selection of the indicated plasmids. Whole-cell extracts were prepared from the harvested cells as previously described (3).

**In vitro synthesis of Ste12p.** Ste12p$^{688}$ and Ste12p$^{214}$ were synthesized by using a rabbit reticulocyte transcription-translation system (Promega). For use as templates in transcription reactions, plasmids pMP101 and pNC292 were linearized by cleavage with *Pst*I. Transcription and translation reactions were performed

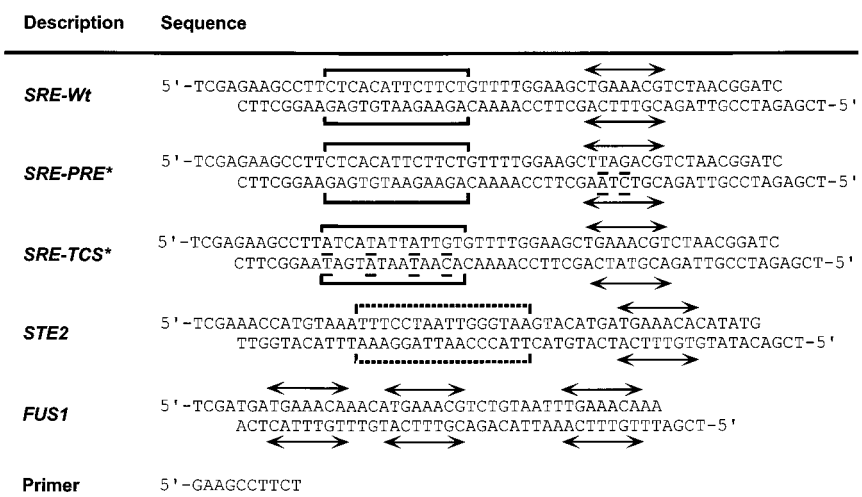


FIG. 2. Ste12p-dependent UAS elements. Oligonucleotides used to assemble the Ty1 SRE, *STE2*, and *FUS1* UASs are shown in their complementary orientations. Also shown is the oligonucleotide primer used for SRE probe DNA synthesis. The sequences delimiting the PRE (↔), the footprint region encompassing the TCS (⌐¬), and the P-box element (:····:) that are binding sites for Ste12p, a Ste12p-associated factor, and Mcm1p, respectively, are indicated.

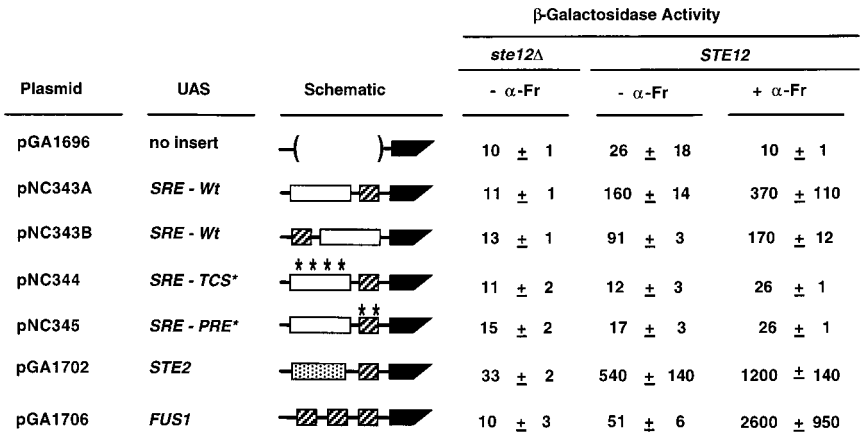


FIG. 3. Comparison of reporter gene expression supported by different Ste12p-dependent UAS elements. The specified UAS elements were inserted at the same position upstream of the *CYC1-lacZ* fusion reporter gene (solid trapezoid). Pertinent features of the different UASs are defined in Fig. 1 and are represented here schematically (PRE, hatched box; TCS, open box; P box, stippled box). Elements with base substitutions are indicated by asterisks. Ste12p dependence of reporter gene expression for each plasmid was assessed by measurement of β-galactosidase activity in *ste12*Δ strain E929-6C-6 and *STE12* strain E929-6C without pheromone induction (− α-Fr). Measurements in *STE12* strain E929-6C were also made after exposure to pheromone (+ α-Fr; 2.5 μM α-factor for 2 h). β-Galactosidase activity is reported in milliunits of optical density at 420 nm per minute per milligram of protein. Values are the average and deviation for measurements made for three independent transformation isolates of each reporter gene plasmid.

under conditions described by Promega. Because the $^{35}$S-labeled proteins interfered with the sensitivity of the gel mobility shift assay, parallel in vitro translation reactions which substituted 1 mM methionine for the [$^{35}$S]methionine were done.

**DNA binding and DNase I footprinting assays.** Gel mobility shift assays for DNA binding activity and DNase I protection assays were performed as described previously (3, 9). Probes for the gel mobility shift assay were prepared by filling in the ends of annealed complementary oligonucleotides or the ends of fragments released from plasmid DNA by restriction enzyme digestion. Fill-in reactions for end labeling of DNA probes were carried out by standard procedures using Klenow polymerase and [α-$^{32}$P]dATP, -dCTP, -dGTP, and -dTTP (33). For DNase I protection assays, probes were end labeled on either the top or the bottom strand. To specifically label the top strand, pNC311 was linearized with *Xba*I, the ends were filled in as before, and the labeled fragment was released by digestion with *Hin*dIII. The bottom strand was similarly labeled except that *Hin*dIII was used to linearize the plasmid and *Xba*I was used to generate the *Hin*dIII-*Xba*I fragment. Labeled DNA fragments were purified by polyacrylamide gel electrophoresis (PAGE) and isolated by the DE-81 method described by Dretzen et al. (8).

**UV cross-linking analysis.** UV cross-linking assays were performed by the method of Chodosh et al. (2), with some modifications. A photoreactive DNA probe that was also uniformly $^{32}$P labeled was synthesized in vitro by using the primer oligonucleotide and complementary SRE-Wt oligonucleotide as the template (Fig. 2). The DNA synthesis reaction mixture contained 6 U of Klenow polymerase, 10 μM dGTP, 10 μM dATP, 10 μM 5-bromo-2-deoxyuridine (BrdU) triphosphate (Sigma), 1 μM dCTP, and 40 μCi of [α-$^{32}$P]dCTP in buffer (7 mM Tris [pH 7.4], 7 mM $MgCl_2$, 50 mM NaCl, 2.5 mM dithiothreitol [Sigma]). Yeast extracts were incubated with the photoreactive probe under standard conditions for DNA-protein complex formation except that the reactions were scaled up 10-fold compared with those for gel mobility shift assays. UV treatment for protein-DNA cross-linking was carried out for 10 min at a distance of 2 in. from a germicidal lamp. Electrophoresis conditions were identical to those described previously for gel mobility shift assays (3). After electrophoresis, gels were covered with Saran Wrap and exposed to X-ray film for 2 h so that cross-linked, shifted protein-DNA complexes could be located and excised from the gel. The gel slices were wedged into a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel either directly or after soaking in DNase I buffer (40 mM Tris [pH 7.5]–6 mM $MgCl_2$ with 400 ng of DNase I [Sigma]) for 60 min. SDS-sample buffer (125 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue) was loaded on top of the gel slices in the SDS-polyacrylamide gel. Electrophoresis conditions were 1 h at 50 V and 4 h at 150 V. After electrophoresis, gels were stained with Coomassie blue (Sigma), and destained to determine the positions of molecular weight standards. Gels were dried and exposed to X-ray film with an intensifying screen at −70°C.

## RESULTS

**Sequence requirements for Ty1 SRE function.** To establish which regions of the Ty1 SRE are critical for its UAS function, we compared reporter gene expression supported by the wild-type SRE (SRE-Wt) with derivatives that have base pair substitutions in either the PRE or TCS site (SRE-PRE* or SRE-TCS*, respectively). The different synthetic elements were inserted at the same position in the promoter-disabled *lacZ* reporter plasmid, pGA1696 (Fig. 2 and 3) (9). UAS function of the different derivatives was then determined by measuring activity of the *lacZ* product, β-galactosidase, in cells carrying the different reporter gene plasmids. Depending on the orientation, the SRE-Wt UAS caused a 3.5- to 6-fold activation of reporter gene expression compared with the control lacking a UAS (Fig. 3, pNC343A, pNC343B, and pGA1696). Base substitutions within either the PRE or the TCS reduced β-galactosidase activity to near background amounts (Fig. 3, pNC345, pNC344, and pGA1696). Because the PRE and TCS are both essential for Ty1 SRE UAS activity, the two regions might function interdependently.

**Comparison of Ty1-SRE, *STE2*, and *FUS1* UAS activities.** To evaluate the Ty1 SRE relative to other Ste12p-dependent elements in a standardized system, we compared the activity of the SRE with those of the *FUS1* and *STE2* UASs when inserted in the same position of the promoter-disabled *lacZ* reporter plasmid, pGA1696. The latter two UASs differ from the Ty1 SRE in that the minimal *FUS1* UAS is comprised of three PREs and the minimal *STE2* UAS has one PRE and an associated P site for binding to Mcm1p (Fig. 2 and 3). Under vegetative conditions (*STE12*, without α-factor), each UAS had a different potency of reporter gene expression relative to the pGA1696 control (Fig. 3). This relative activation was 20-fold for the *STE2* UAS (pGA1702), 6- or 3.5-fold for the Ty1 SRE, depending on orientation (pNC343A and -B), and only 2-fold for the *FUS1* UAS (pGA1706). Despite these quantitative differences, under vegetative growth conditions, all three UASs showed a similar dependence on Ste12p. The Ty1 SRE supported 7- or 15-fold (depending on orientation) more β-galactosidase activity in *STE12* cells relative to *ste12*Δ cells (Fig. 3, pNC343B or pNC343A). The *STE2* (16-fold) and *FUS1* (5-fold) UAS elements showed similar ranges of dependency (Fig. 3, pGA1702 and pGA1706, respectively).

Ste12p is best characterized for its role in pheromone-induced transcriptional activation. Therefore, we also compared the Ty1 SRE, *STE2*, and *FUS1* UAS elements for reporter

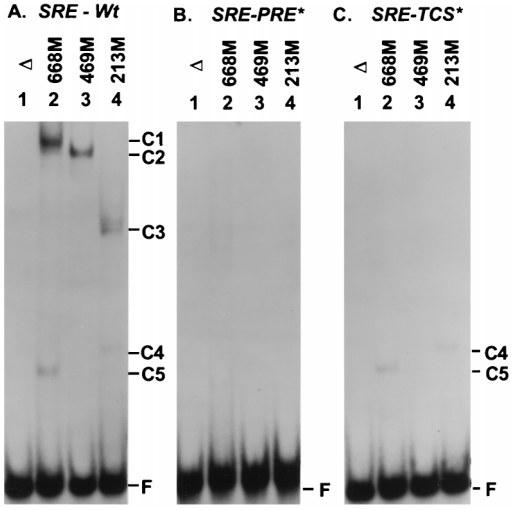


FIG. 4. Ste12p-dependent complex formation with Ty1 SRE DNA probes. Protein extracts (25 μg) from a *ste12*Δ yeast strain (E929-6C-6) containing no Ste12p (Δ; pNC160), $Ste12Mp^{668}$ (668M; pNC248), $Ste12Mp^{469}$ (469M; pGA1635), or $Ste12Mp^{213}$ (213M, pGA1841) were used in gel mobility shift assays with SRE-Wt or a substitution mutation (SRE-PRE* or SRE-TCS*) probe as indicated. The positions of free probe (F) and DNA binding complexes (C1, C2, C3, C4, and C5) are indicated at the right of each panel.

gene activation under pheromone-induced conditions. Even though the three elements show similar dependence on Ste12p, they do not show similar responses to pheromone induction. Treatment of cultures with α-factor resulted in a 50-fold induction of β-galactosidase activity with the *FUS1* UAS (Fig. 3, pGA1706). By contrast, the Ty1 SRE and *STE2* UASs showed only a twofold induction (Fig. 3, pNC343A, pNC343B, and pGA1702). Interestingly, the natural *STE2* promoter includes two PREs in addition to the one present in the minimal UAS analyzed here (26). Within the context of the natural promoter, Ste12p mediates a four- to five-fold induction of *STE2* expression by pheromone (11, 14). These various differences in vegetative and pheromone-induced activities highlight the quantitatively and qualitatively distinct modes of transcriptional regulation mediated by Ste12p, depending on the context of the PREs.

**Protein complex formation with SRE and substitution derivative DNA probes.** To investigate the roles of the PRE and TCS sites in protein-DNA complex formation, the wild-type (SRE-Wt) and substitution sequence (SRE-PRE* and SRE-TCS*) probes were compared in gel mobility shift assays (Fig. 2 and 4). One major Ste12p-dependent complex (C1) formed with the SRE-Wt probe, using protein extracts from cells expressing $Ste12Mp^{668}$ (Fig. 4A, lane 2). Consistent with the known presence of Ste12p in this complex, the mobility of this DNA-protein complex increased (positions C2 and C3) when we used extracts from cells expressing the $Ste12Mp^{469}$ or $Ste12Mp^{213}$ (Fig. 1; Fig. 4A, lanes 3 and 4) (9). Complexes C1, C2, and C3 were not observed with Ste12p or its truncation derivatives when either the SRE-PRE* or SRE-TCS* substitution probe was used (Fig. 4B and C). Therefore, both sequence elements are required for formation of the different-mobility complexes that form with $Ste12Mp^{668}$ or its truncated derivatives.

Extracts from cells expressing $Ste12Mp^{213}$ yielded a second, weaker complex (C4) with a mobility closer to that of the free probe (Fig. 4A, lane 4). Because complex C4 is present with the SRE-TCS* probe but not the SRE-PRE* probe (Fig. 4B and C, lane 4), complex C4 is attributed to the occupancy of the PRE binding site by $Ste12Mp^{213}$ without associated binding at the TCS. This interpretation was confirmed by DNase I protection analyses (see below). By analogy, the weak complex seen with the $Ste12Mp^{668}$ extract at position C5 is attributed to binding of a Ste12p degradation product that is comparable to $Ste12Mp^{213}$.

**Protein binding sites in the Ty1 SRE.** Knowledge of the DNA binding sequences in any protein-DNA complex establishes parameters useful for the identification of proteins in the complex. Such information also was pertinent to interpretation of other experiments that we implemented to deduce which proteins in the Ty1 SRE complex contribute to transcriptional activation (see below). With these objectives in mind, we used DNase I protection assays to define the SRE sequences involved not only in complex C1 but also in complexes C3 and C4. The assays were performed with end-labeled SRE probes, using extracts from yeast cells expressing $Ste12Mp^{668}$ for complex C1 formation or $Ste12Mp^{213}$ for complex C3 and C4 formation (Fig. 5). DNA retained in complex C1 (668M) and complex C3 (213M) showed protections from DNase I cleavage located in the PRE and TCS regions of the probe (Fig. 5A and B, lanes 4 and 6). Further, the footprints are virtually identical for two complexes. The simplest interpretation of this outcome is that the same proteins (or their DNA binding domains) are in contact with the DNA in the two complexes. Another implication is that the DNA binding domain of Ste12p is sufficient to promote the protein interactions that involve the TCS. By contrast, DNA retained in complex C4 (213M) showed protections in the PRE but not the TCS (Fig. 5B, lane 10). These results are consistent with the expectation that Ste12p binds to the PRE sequence within the SRE and that another protein binds at the TCS.

**SRE-protein complex formation requires components in yeast extracts in addition to Ste12p.** In vitro-synthesized $Ste12p^{688}$ was used in gel mobility assays to learn whether Ste12p is sufficient for complex C1 formation (Fig. 1 and 6A). $Ste12p^{688}$ produced from the reticulocyte lysate system (R-688) did not form any complex under the conditions of our binding reactions (Fig. 6, lane 3). However, binding was reconstituted by the addition of extract from the ste12Δ strain (YeΔ) to the in vitro-synthesized preparation (Fig. 6, lane 4). In contrast to the much sharper complex that forms with $Ste12pM^{668}$ from yeast (Y-668M), there is a heterogeneous mixture of complexes formed in the reconstituted system (Fig. 6B, lanes 4 and 5). The distribution can be attributed, at least in part, to the size heterogeneity of in vitro-synthesized Ste12p (Fig. 6A, lane 3). We speculate that the skewed abundance of complexes with higher mobility than C1 reflects a preference for complex formation with certain of the truncated derivatives present in the preparation. Nevertheless, for all of these reconstituted complexes, the yeast extract presumably provides either a binding partner or modifying enzyme that makes $Ste12p^{688}$ (or its truncation derivative) competent to bind the SRE.

Gel shift and DNase I protection assays were also performed with in vitro-synthesized $Ste12p^{214}$. Unlike the full-length protein, the truncated $Ste12p^{214}$ formed a complex with the SRE probe. However, in contrast to the two complexes (C3 and C4) seen with $Ste12Mp^{213}$ from yeast (Y-213M), only one complex (C4′) was detected with the reticulocyte synthesized $Ste12p^{214}$ (R-214) (Fig. 6B, lanes 6 and 8). (The mobility difference between complex C4′, resulting from in vitro-synthesized $Ste12p^{214}$, and complex C4, resulting from $Ste12Mp^{213}$ from yeast, is accounted for by the 10 amino acids contributed by the Myc epitope tag.) Formation of the second complex (C3) was observed only when extract from the *ste12*Δ strain (YeΔ) was

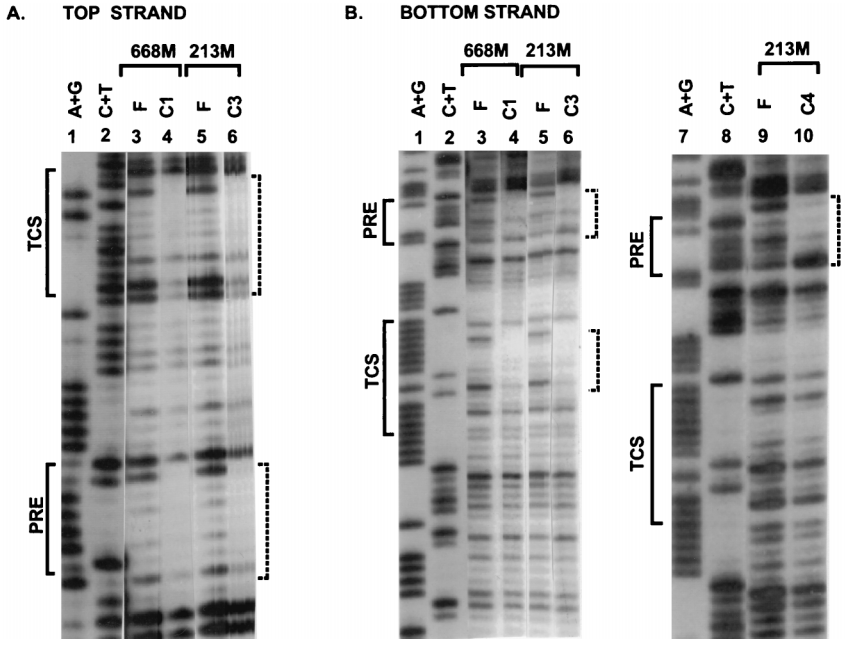


FIG. 5. DNase I protection analysis of Ste12p-dependent Ty1 SRE protein complexes. Binding reactions contained Ty1 SRE probe DNA that was end labeled on the top strand (A) or bottom strand (B) and yeast protein extracts containing Ste12Mp$^{668}$ (668M) or Ste12Mp$^{213}$ (213M) as indicated. Lanes display the DNase I cleavage products for free probe (F) and DNA-protein complex C1, C3, or C4 (Fig. 4) as specified. Markers (A+G and C+T) were generated from probe DNA by Maxam-Gilbert purine-specific and pyrimidine-specific reactions. Solid brackets to the left delimit regions corresponding to the PRE and TCS. Dashed brackets to the right delimit sequences protected from DNase I cleavage.

added to the binding reaction (Fig. 6B, lane 7). This finding suggests that complex formation with the Ty1 SRE requires a distinct binding partner for Ste12 or a modified form of Ste12p that is competent for binding to both the PRE and TCS.

**UV cross-linking analysis of proteins binding the Ty1 SRE.** DNA-protein cross-linking was used to test whether proteins in addition to Ste12p bind to the Ty1 SRE. In this assay proteins in direct contact with the DNA probe were cross-linked to [$^{32}$P]BrdU-labeled DNA by using UV irradiation. The indirectly labeled species were then fractionated by SDS-PAGE and visualized by autoradiography.

Three sizes of cross-linked species were observed in the analysis of proteins from Ste12Mp$^{668}$ extracts that form complex C1. These are at positions A (175 kDa), B (135 kDa), and D (90 kDa) (Fig. 7, lane 1). To decrease the amount of DNA in each linkage and get a closer estimate of protein sizes, a gel slice containing the cross-linked complex was incubated with DNase I prior to SDS-PAGE. Although DNase I digestion was incomplete, the signal at position A disappeared and a portion of the cross-linked species migrated to higher-mobility positions B* (115 kDa) and D* (72 kDa) (Fig. 7, lane 2). The large size of the species at position A and the fact that it disappeared after DNase I treatment suggests that this signal could have arisen from two proteins (B and D) tethered together by the DNA probe. Because Ste12Mp$^{668}$ has an apparent size of 116 kDa on SDS-PAGE, it is likely that the signals at positions B and B* are [$^{32}$P]DNA cross-linked to Ste12Mp$^{668}$. The signals at positions D and D* could represent either a Ste12p degradation product or distinct protein that binds to the SRE.

To confirm that the signal at position B is from Ste12Mp$^{668}$ and to distinguish whether the signal at position D is from a Ste12Mp degradation product or a distinct protein, the UV cross-linking assay was applied to complex C3, which forms with extracts from cells expressing the much smaller Ste12Mp$^{213}$. These analyses revealed four signals at positions C (120 kDa), D (90 kDa), E (55 kDa), and F (46 kDa) (Fig. 7, lane 3). As before, we conjecture that species C arose from two proteins tethered together by DNA. The absence of species B and the appearance of cross-linked proteins at positions E and F are consistent with our assignment of B as the [$^{32}$P]DNA-

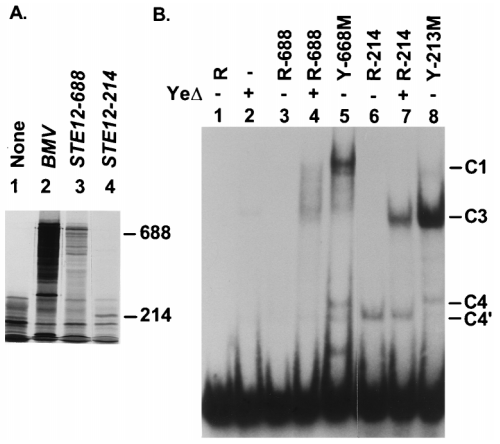


FIG. 6. Gel mobility shift assays using in vitro-synthesized Ste12p or Ste12p DNA binding domain. (A) SDS-PAGE analysis of [$^{35}$S]methionine-containing products from coupled transcription-translation reactions with template RNA as follows: lane 1, no RNA; lane 2, brome mosaic virus (BMV) control RNA; lane 3, *STE12-688* mRNA (transcribed from pM101); lane 4, *STE12-214* mRNA (transcribed from pNC292). The positions of Ste12p$^{688}$ (688) and Ste12p$^{214}$ (214) are indicated to the right. (B) Gel mobility shift assays comparing SRE complex formation with in vitro synthesized Ste12p$^{688}$ (R-688) or Ste12p$^{214}$ (R-214) in the absence (−) or presence of (+) extract from *ste12*Δ strain E929-6C-7 (ΔY), as indicated. Binding activities of yeast extracts from cells expressing Ste12Mp$^{668}$ (Y-668M) or Ste12Mp$^{213}$ (Y-213M) are included for reference. The positions of DNA binding complexes C1, C2, C3, C4 and C4′ are indicated to the right.

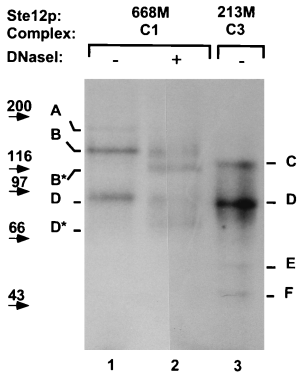


FIG. 7. SDS-polyacrylamide gels displaying proteins cross-linked to $^{32}$P-labeled DNA in Ste12p-dependent SRE complexes. Binding reactions for the cross-linking analysis used yeast protein extracts (200 μg) from strains expressing either Ste12Mp$^{668}$ (E929-6C-6 pNC247) or Ste12Mp$^{213}$ (E929-6C-6 pGA1841) and a uniformly $^{32}$P-labeled BrdU SRE probe. Slices from mobility shift gels containing Ste12Mp$^{668}$-dependent complex C1 (668M/C1) or Ste12Mp$^{213}$-dependent complex C3 (213M/C3) were subjected to SDS-PAGE without (−) or with (+) DNase I treatment (60 min, 400 ng). Different-size cross-linked protein species are labeled (A, 175 kDa; B, 135 kDa; B*, 115 kDa; C, 120 kDa; D, 90 kDa; D*, 72 kDa; E, 55 kDa; F, 46 kDa.). Arrows to the left indicate positions of size markers (in kilodaltons).

cross-linked Ste12p$^{668}$ in complex C1. Signals from complex C3 that appear at positions E and F most likely arise from Ste12Mp$^{213}$ linked to two different-size DNA fragments. (Such heterogeneity easily could result from fortuitous nucleolytic cleavage during handling of the samples.) Nevertheless, the critical finding is that the signal at position D is the only species that is common to both complexes C1 and C3 (Fig. 7, lanes 1 and 3). This outcome rules out the possibility that species D arises from a degradation product of Ste12p and supports the interpretation that the cross-linked species is a distinct SRE binding protein.

**Ste12p contributes the transcriptional activation domain(s) associated with UAS function of the Ty1 SRE.** Ste12Mp$^{213}$ retains the DNA binding domain of Ste12p but is missing the domains required for transcriptional activation (38), yet Ste12Mp$^{213}$ still recruits the cooperative binding protein that protects the TCS region of the Ty1 SRE (Fig. 5 to 7). Therefore, measurement of reporter gene expression in a *ste12*Δ strain that expresses Ste12Mp$^{213}$ should reveal the contribution, if any, of the cooperative protein to transcriptional activation mediated by the Ty1 SRE. Compared with the vector and Ste12Mp$^{668}$ reference strains, there was only a background amount of β-galactosidase expressed from the *FUS1-lacZ* reporter gene in the Ste12Mp$^{213}$ strain (Fig. 8, pGA1706). There was weak stimulation of the *STE2-lacZ* reporter gene in the Ste12Mp$^{213}$ strain (Fig. 8, pGA1702). This residual *STE2* UAS activity has been attributed to Mcm1p, which is a weak transcriptional activator and binds to the *STE2* UAS independently of Ste12p (9, 15). There was no significant residual activity of the SRE-*lacZ* reporter gene in the Ste12Mp$^{213}$ strain (Fig. 8 pNC343). Thus, it appears that at least in the context of this ~50-bp SRE, the TCS binding factor is not a transcriptional activator on its own. Instead, its role appears to be essential for cooperative binding with Ste12p.

**SRE UAS function is dependent on Ste12p and Tec1p.** Its been shown that Ty1 SRE reporter gene expression is reduced to background amounts in strains lacking either Ste12p or Tec1p (3, 22). To learn if this dependence was direct, we exploited a Tec1-Gal4p$^{AD}$ fusion that functions as a one-hybrid transcriptional activator (6). This hybrid stimulated expression of the *lacZ* reporter under control of SRE-Wt (Fig. 9, pNC343A and pNC343B). It also stimulated expression of the *lacZ* reporter under control of the PRE*-SRE that has base substitutions in the Ste12p binding site (Fig. 9, pNC345). However, it did not stimulate reporter expression under control of the TCS*-SRE that has mutations in the binding site for the protein that cooperates with Ste12p for binding to the SRE (Fig. 9, pNC344). The results with the Tec1-Gal4p$^{AD}$ hybrid are consistent with the interpretation that Tec1p either binds directly to the Ty1 SRE or binds to another protein that does.

Comparison of SRE-protein complex formation in gel mobility shift assays using extracts from wild-type, *ste12*Δ, and *tec1*Δ strains showed that both Tec1p and Ste12p are necessary for complex C1 formation (Fig. 10, lanes 1 to 3). To test if Tec1p is physically present in the protein-SRE complex, we used extracts from a strain that expressed Tec1-Gal4p$^{AD}$ in the gel mobility shift assay. The presence of two higher-mobility complexes (C1′ and C1″) in binding reactions with Tec1p-Gal4p$^{AD}$ extracts confirms that the hybrid, which is smaller than Tec1p, is part of the complex (Fig. 10, lane 4). Because the signals are diffuse, we believe that the complexes with the Tec1-Gal4p$^{AD}$ hybrid are relatively unstable under our standard in vitro binding assay conditions. This implied instability and the difference between in vitro and in vivo conditions could

| Plasmid | UAS | Schematic | α-Fr | β-Galactosidase Activity: No Ste12p | Ste12Mp$^{213}$ | Ste12Mp$^{668}$ |
|---|---|---|---|---|---|---|
| pGA1696 | no insert | | - | 2 ± 1 | 1 ± 1 | 15 ± 6 |
| pNC343A | *SRE - Wt* | | - | 2 ± 1 | 11 ± 5 | 1100 ± 290 |
| | | | + | 2 ± 1 | 5 ± 3 | 2900 ± 150 |
| pNC344 | *SRE - TCS** | | - | 3 ± 1 | 2 ± 1 | 19 ± 3 |
| pNC345 | *SRE - PRE** | | - | 5 ± 2 | 1 ± 1 | 25 ± 10 |
| pGA1702 | *STE2* | | - | 21 ± 3 | 42 ± 12 | 2000 ± 950 |
| pGA1706 | *FUS1* | | - | 2 ± 1 | 3 ± 1 | 190 ± 15 |
| | | | + | 2 ± 1 | 1 ± 1 | 8200 ± 310 |

FIG. 8. Comparison of reporter gene expression in strains expressing Ste12Mp$^{213}$ or Ste12Mp$^{668}$. Reporter gene expression for each plasmid was assessed by measurement of β-galactosidase activity in *ste12*Δ strain E929-6C-6 expressing either no Ste12p (pNC160), Ste12Mp$^{668}$ (pNC247), or Ste12Mp$^{213}$ (pGA1841). The different UAS elements inserted upstream of the *CYC1-lacZ* reporter are as defined in Fig. 1. β-Galactosidase activity is reported in milliunits of optical density at 420 nm per minute per milligram of protein. Values are the average and deviation for measurements made for three independent transformation isolates of each reporter gene plasmid.

| Plasmid | UAS | Schematic | β-Galactosidase Activity: Vector | Tec1-Gal4p$^{AD}$ |
|---|---|---|---|---|
| pNC343A | *SRE - Wt* | | 88 ± 13 | 850 ± 280 |
| pNC343B | *SRE - Wt* | | 92 ± 18 | 1540 ± 110 |
| pNC344 | *SRE - TCS** | | 9 ± 3 | 7 ± 3 |
| pNC345 | *SRE - PRE** | | 15 ± 2 | 2970 ± 130 |

FIG. 9. Tec1-Gal4p-dependent activation of SRE-mediated reporter gene expression. Reporter gene expression for each plasmid was assessed by measurement of β-galactosidase activity in *STE12* strain E929-6C expressing Tec1-Gal4p$^{AD}$ (pMHO4) or not (vector; pACT). The different UAS elements inserted upstream of the *CYC1-lacZ* reporter are as defined in Fig. 1. β-galactosidase activity is reported in milliunits of optical density at 420 nm per minute per milligram of protein. Values are the average and deviation for measurements made for three independent transformation isolates of each reporter gene plasmid.

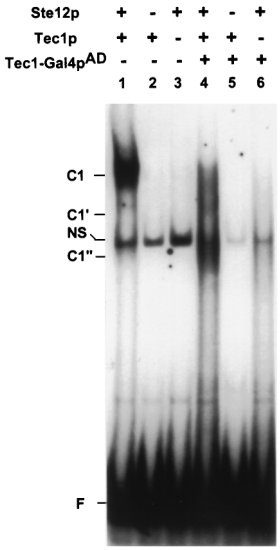


FIG. 10. Gel mobility shift analysis of Tec1p-dependent binding to the Ty1 SRE. Protein extracts (25 μg) from strains E929-6C (*STE12 TEC1*), E929-6C-7 (*ste12-Δ2::URA3 TEC1*), and E929-6C-65 (*STE12 tec1Δ::LEU2*), either expressing (+) or not expressing (−) Ste12p, Tec1p, or Tec1-Gal4p$^{AD}$ (pMOH4), were used in gel mobility shift assays with the wild-type Ty1 SRE DNA probe. The positions of free probe (F) and Ste12p Tec1p-dependent DNA binding complexes (C1, C1′, and C1″) are shown to the left. The position of a nonspecific complex (NS) that is independent of Ste12p and Tec1p is also shown.

readily account for our finding that the Tec1-Gal4p$^{AD}$ hybrid stimulated reporter gene expression from the SRE-PRE* UAS, which does not have a functional Ste12p binding site, but failed to form a detectable protein-DNA complex from extracts lacking Ste12p. Nevertheless, the mobility pattern of complexes C1, C1′, and C1″ suggests that their formation with Tec1-Gal4p$^{AD}$ in vitro is likely to involve Ste12p and different heteromultimers of Tec1p and Tec1-Gal4p$^{AD}$.

## DISCUSSION

**Ste12p and a 72-kDa protein bind cooperatively to the Ty1 SRE.** The Ty1 SRE was originally identified as a region of the transposable element that was sufficient to support Ste12p-dependent activation of adjacent gene expression and protein-DNA complex formation (3). It subsequently was found also to depend on Tec1p for its UAS activity (22). Analysis of SRE base substitution derivatives helped to define two regions, called the PRE and TCS, that are required for its function (references 3, 22, and 24 and this work). The PRE is well established as a binding site for Ste12p. UV cross-linking experiments confirmed that a second protein with an apparent size of 72 kDa also directly contacts the Ty1 SRE DNA. Based on the footprint of SRE-protein complexes, we deduce that this second protein binds to the TCS region of the DNA.

Two observations lead to the conclusion that the binding interactions of Ste12p and the 72-kDa protein are cooperative. First, base substitutions in either the PRE or TCS abolish all protein complex formation. Second, neither protein alone forms a complex that gel mobility shift assays can detect. There is no observable SRE-protein complex either in assays using extract from a *ste12*Δ strain or in assays using in vitro-synthesized full-length Ste12p (Ste12p$^{688}$), yet binding is reconstituted by the addition of yeast extract from a *ste12*Δ strain to the in vitro-synthesized Ste12p. Interestingly, the DNA binding domain of Ste12p (residues 1 to 213) appears to be sufficient for the cooperative interaction with the 72-kDa protein because a protein complex with footprints in both the PRE and TCS regions forms with Ste12Mp$^{213}$ yeast extract. A complex of the same mobility also forms by reconstitution of an extract from a *ste12*Δ strain with in vitro-synthesized Ste12p$^{214}$.

In this cooperative interaction, the TCS binding protein appears to make Ste12p competent to bind its site in the SRE, while Ste12p provides the activation function of the complex. Other studies have shown that the central domain of Ste12p (residues 214 to 461) is its activation domain (38). Although Ste12Mp$^{213}$, which lacks this activation domain, still forms an SRE complex with the TCS binding protein, there is no transcriptional activation of the Ty1 SRE-*lacZ* reporter gene. Therefore, in the context of the Ty1 SRE, the TCS binding protein appears not to be a transcriptional activator on its own.

**Is Tec1p the TCS binding protein?** Previous genetic and biochemical observations make a strong argument that Tec1p is the TCS binding protein. Ty1 SRE function as a UAS is dependent on both Ste12p and Tec1p (3, 22). We show here that both gene products are also required for SRE complex formation with proteins from yeast whole-cell extracts. Gel mobility shift experiments with extracts that contained a Tec1-Gal4p$^{AD}$ fusion protein further showed that Tec1p is physically present in the SRE-protein complex and therefore has a direct role in SRE protein complex formation. Because Tec1p has a predicted TAE DNA binding domain, it is probable that Tec1p directly binds to the TCS region of the Ty1 SRE. Still, our experiments do not rule out the caveat that Tec1p may be a part of the SRE complex through protein-protein interactions. However, experiments of Madhani and Fink (24) which were published after submission of this report rule out the alternative explanation. Their study showed that maltose-binding protein fusions of Ste12p and Tec1p purified from *Escherichia coli* were sufficient to reconstitute a protein complex with the Ty1 SRE probe.

**The SRE is a composite regulatory element that defines a distinct Ste12p binding specificity.** Similar to the situation at the *STE2* UAS, Ste12p binding to a single PRE sequence is entirely dependent on the occupancy of an adjacent recognition element by a second protein. In the case of the *STE2* UAS, the adjacent site is a P box which binds to Mcm1p. Whereas Mcm1p binds to its site independently of whether Ste12p occupies the PRE, Tec1p requires the reciprocal occupancy of Ste12p at the PRE (references 9 and 24 and this work). In this sense, the TCS and PRE function as a single entity with a unique recognition specificity. This combinatorial regulatory element also has transcriptional properties that are distinct from those of other PRE-containing UASs. For example, despite being highly Ste12p dependent, this combinatorial regulatory element is not pheromone inducible and clearly alters the transcriptional activation properties of Ste12p compared with its action in other sequence contexts (Fig. 3 and references 13, 18, and 20). Such combinatorial specificity and context dependence of Ste12p action readily explain how this transcription factor can mediate changes in gene expression that are crucial for very distinctive cellular events such as mating and pseudohyphal differentiation.

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